ANTIGENIC DIFFERENCES BETWEEN STEM AND HYDRANTH IN TUBULARIA [1]

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The marine hydroid, *Tubularia crocea,* consists essentially of two layers of tissue which are differentiated into a hydranth with hypostome, two groups of tentacles and gonophores, and a stem portion surrounded by a chitinous perisarc. Chemical differences may underlie these morphological differences between the hydranth and stem regions. Since the morphallactic regeneration of a hydranth from the stem region involves a remodeling of a certain portion of the stem tissues, further chemical changes may accompany visible morphological changes during regeneration of a hydranth. Because proteins and other complex molecules are the principal substances of organic forms, basic changes in morphogenesis probably involve production and transformation of such molecules and their aggregates.

The key role presumably played by the metabolism of complex molecules and especially proteins has interested developmental biologists for some time. A number of workers have used immunological methods to study differentiation of such substances and to correlate changes in their composition with visible morphological differentiation [reviewed by Irwin (1949), Ebert (1955), Tyler (1955, 1957), Woerdeman (1955), Schechtman (1955), Nace (1955), and Brachet (1957)]. While there have been many immunochemical studies concerned with embryonic development, immunological methods have rarely been used in studying problems of regeneration although the usefulness of such methods in studies on regeneration has been mentioned by Woerdeman (1953). De Haan (1956) and Laufer (1957) have used immunological techniques to study muscle differentiation in the regenerating limb of axolotl larvae.

In spite of numerous investigations on regeneration in *Tubularia* there is little information on the chemistry of this organism. A rational prerequisite to the investigation of chemical changes during hydranth regeneration would seem to involve the determination of chemical differences between the two main regions, the hydranth and the stem. Accordingly, the present study was undertaken to determine the similarities and differences in the antigenic composition of *Tubularia crocea* hydranths and stems (*cf.* preliminary note by Morrill, 1958).

Materials and Methods

A species of *Tubularia, T. crocea,* was collected from the St. John's River jetties in Florida, where the organism is abundant from October to June. Only

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colonies whose stems were relatively free of macroscopic epizooites were used. After their collection the animals were kept in jars of sea water for not more than 36 hours.

Clean whole animals, stems, and hydranths were frozen in a dry-ice bath. Hydranths and stems were prepared by first cutting off the hydranths and then removing the distal 4 millimeters of the stem plus any basal region of the stem that had epizooites. Stems were frozen within 20 minutes after hydranth removal. Hydranths were frozen within 4 hours after they had been severed from the stems. Separation of hydranths and stems on a sexual basis proved to be impractical. The frozen tissues were lyophilized and stored at − 20° C.

Preparation of saline extracts for use as antigens

One-gram quantities of lyophilized tissue were ground in a mortar and extracted with 10 ml. of buffered saline (9 g. NaCl/l., 0.1 *M* phosphate buffer, pH 7.0) for 8 to 12 hours at 4 to 10° C. At the end of this period the supension was homogenized in a glass homogenizer in an ice bath. The homogenate was kept at 4 to 10° C. for two more hours. Then the homogenate was centrifuged for 30 minutes at a centrifugal pressure of approximately 4500 *g* and the cloudy supernatant collected and stored at − 20° C. The nitrogen content of the extracts, determined by a modified nesselerization method (Hawk *et al.*, 1954), varied as follows: whole animal, 0.15 to 0.50 mg. N/ml.; hydranth, 0.25 to 0.82 mg. N/ml.; stem, 0.23 to 0.68 mg. N/ml.

The degree of contamination with insoluble particulate matter may have varied in the extracts. Therefore, equilibration on a nitrogen basis is at best an approximate equalization of antigen concentration.

Preparation of antisera

Nine male rabbits were used in the preparation of antisera. None of the preinjection rabbit sera precipitated the extracts. Several injection routes were employed. These included intravenous, intermuscular with oil emulsion adjuvant (Freund, 1947), and intraperitoneal injections. The intraperitoneal route yielded the most satisfactory antisera. Two rabbits received three series of intraperitoneal injections over a two-month period. Each series consisted of 10 to 15 milliliters of whole animal extract administered within 6 to 8 days. The extracts injected into these rabbits were not adjusted on a nitrogen basis. One of these rabbits (Rabbit 4) was injected with the supernatant fraction of the homogenate. The other (Rabbit 6) was injected with the entire homogenate.

Blood was collected in sterile 50-milliliter centrifuge tubes 5, 7, and 17 days after the last injection of each series and allowed to clot at room temperature for 4 to 5 hours. The clots were loosened and the tubes stored at 4 to 10° C. for 24 hours. The serum then was decanted, centrifuged, and stored in vials at − 20° C. until used.

Serological tests

In order to test for antigenic differences between hydranth and stem tissues two types of precipitin tests were used. The first test employed was the standard interfacial ring test. When this test failed to reveal any antigenic differences, even when antiserum absorbed with hydranth or stem extract was used, the Ouchterlony

agar gel diffusion method was employed in order to determine the spectrum of individual precipitin reactions and what differences might exist between the spectra of precipitin lines produced by anti-whole animal serum and stem and hydranth extracts.

Interfacial ring tests were performed by layering 0.05 ml. *Tubularia* extract over 0.05 ml. antiserum or over saline or pre-injection serum controls in 3 × 20 mm. capillary tubes. The tubes were capped with plastocene clay, incubated at 37° C., and examined after one and two hours for the presence of a precipitating ring at the interfaces. The highest antiserum titer (dilution of antiserum) of the sera from the two rabbits was 64. The highest antigen titer of the saline extracts was 16,000. These titers were obtained with the sera collected 5 and 7 days after the third injection series. No precipitates formed in control tests where pre-injection serum plus saline extracts and antiserum plus saline alone were employed.

The agar gel diffusion technique of Ouchterlony (Ouchterlony, 1949) was employed with modifications by Nace (personal communication). Details of the method are as follows. Four per cent agar was prepared and washed in distilled water for several days. This was used to prepare an aqueous 2 per cent agar containing aqueous merthiolate (0.25 ppth) and methyl orange (40 mg. per 500 ml. agar). A basal layer of this melted 2 per cent agar was poured on the bottom of petri dishes. After this layer had hardened additional agar was added and a lucite well mold placed in position. When the agar had hardened the mold was removed.

The wells were filled with antigens and antisera—0.30 ml. in the large square well and 0.15 ml. in the narrow rectangular wells. The center well of each plate was filled with antiserum or pre-injection serum and the four surrounding wells with saline extracts or saline. The plates were incubated in high humidity in an air-tight container at 37° C. for 7 days. They were then brought to room temperature for 6 to 24 hours and finally left at 5° C. for an additional 7 days.

At the end of 14 days the agar plates were fixed in 5 per cent formalin with methyl orange added, mounted between thin glass plates, and inserted into a photographic enlarger. The focused image of the wells and precipitin lines was so faint, particularly in the region of coalescence of lines, that photographing the preparations proved to be impractical. Therefore, the enlarged diagram of the lines was recorded by tracing on paper .

The antisera were absorbed in the following way: antisera and saline extracts or saline controls were mixed in various proportions in small sterile tubes, incubated at room temperature for two hours, placed in the refrigerator for 36 to 48 hours, and centrifuged. The supernatant was then subjected to interfacial ring tests and agar gel diffusion tests.

Results

In order to test for differences in the antigenic composition of *Tubularia* hydranths and stems three tests were employed—the precipitin ring test, the Ouchterlony agar gel diffusion test, and the absorption test which was used in conjunction with the other two tests.

Precipitin ring tests

Precipitin antigen titers were determined in order to test for quantitative and qualitative differences between stem and hydranth extracts. Such extracts were

equilibrated on a nitrogen basis, serially diluted and layered over anti-whole animal antiserum. No differences in the titers were observed. Evidently the two types of extracts were similar on a gross quantitative basis. To test the possibility of the existence of qualitative differences, absorption experiments were performed. Partial absorption of anti-whole animal antiserum by either extract produced antiserum which still reacted equally with both types of extract; complete absorption by either extract produced sera which failed to form a precipitin ring with either extract. The precipitin ring tests then failed to reveal any distinct antigenic differences between hydranth and stem extracts.

Because these tests failed to reveal any antigenic differences, it seemed desirable to utilize a method where the precipitate of the precipitin ring could be separated into a spectrum of one or more precipitin reactions. Accordingly, the antigenic composition of the extracts was examined by means of the agar gel diffusion technique of Ouchterlony.

Ouchterlony tests

Tests with unabsorbed sera. Preliminary Ouchterlony tests of anti-whole animal sera revealed multiplicity of precipitating systems. Similarities as well as differences existed between the precipitin patterns with stem and hydranth extracts. As expected, the number of lines formed varied with the antisera from the different rabbits. No precipitin lines appeared with pre-injection sera and saline controls. It was found that the pattern of precipitin lines varied in repeated tests with a given antiserum, possibly because the saline extracts used as test antigens were prepared at different times, even though the extracts were prepared by a standard procedure and adjusted on a nitrogen basis. In addition, antisera from a rabbit taken at different times following a series of injections exhibited variations in position and in number of lines in the precipitate patterns when tested with hydranth and stem extracts. This is probably because the maximum concentration of antibodies for the several antigens did not occur at the same time (Abramoff and Wolfe, 1956). The most complete precipitin line patterns were obtained with antisera from Rabbit 4 and Rabbit 6 (see under Methods) obtained 5 and 7 days after the third series of intraperitoneal injections.

The best precipitin pattern produced by anti-whole animal sera with hydranth and stem extracts is given in Figure 1. This antiserum from Rabbit 4 produced a total of seven precipitin lines with hydranth extract and seven precipitin lines with stem extract. Six precipitin lines with hydranth extract coalesced with five lines produced with stem extract. One additional line was restricted to the hydranth extract. Two precipitin lines and a spur on a coalescing line were restricted to the stem extract. Fewer lines were produced with antiserum of Rabbit 6. In the best pattern with antiserum from this rabbit four lines produced by hydranth extract coalesced with three lines formed by hydranth extract. In addition two precipitin lines were limited to reactions with components of stem extract, and possibly one was limited to hydranth extract.

In the several experiments the coalescing lines formed complex patterns. In addition to the fusion of single lines formed by the two extracts, there were instances where two hydranth precipitin lines coalesced with one line formed by stem extract and vice versa. These results may be interpreted as being due to superimposed

precipitin lines in the reaction of the antiserum with one extract. It is also possible that in one extract an antigenic substance had haptens in common with the haptens of two antigenic substances in the other extract (Kaminski and Ouchterlony, 1951). In nearly all the experiments one of the stem lines that coalesced with a hydranth line had a spur which extended beyond the region of coalescence (Fig. 1). This indicates that this stem antigen had two haptens, one in common with a hydranth antigen and one not found on any of the hydranth antigens. This interpretation is in accord with the explanation for the appearance of spurs given by Kaminski and Ouchterlony (1951).

The large number of lines formed in the Ouchterlony patterns suggested that some of the lines might be due to excessive quantities of certain antigens or antibodies (Kaminski, 1954). It was also possible that one or more of the single lines might be due to several superimposed precipitates (Grasset *et al.*, 1956). Attempts to resolve the complexity of the patterns, however, by diluting either the antisera or

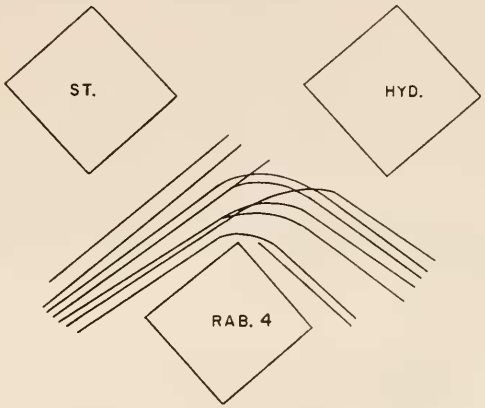

FIGURE 1. Diagram of precipitin pattern of anti-whole animal serum, Rabbit 4, with stem and hydranth saline extracts.

the hydranth and stem extracts resulted in patterns with fewer and more diffuse lines.

The number of lines was also checked by the immunoelectrophoretic method of Grabar and Williams (Grabar and Williams, 1953, 1955). In the first experiment the antigens of whole animal extract were separated in 2 per cent agar electrophoretically (veronal buffer, pH 8.6, ionic strength 0.1, 10 ma., 24 hrs.). After the separation, anti-whole animal serum of Rabbit 4 was placed in a trench parallel to the path of migration and allowed to diffuse into the agar. After seven days' incubation, nine precipitin lines in the form of distinct arcs were detected. In another experiment extracts of hydranths and stems were separated electrophoretically (veronal buffer, pH 8.6, ionic strength 0.05, 30 ma., 5 hrs.) and tested with anti-hydranth serum. This antiserum produced six curving precipitin lines with each extract. The electrophoretic mobilities of the several arcs were similar. This particular antiserum when previously tested by the Ouchterlony method had produced six lines with hydranth extract and five lines with stem extract.

The patterns formed by coalescing lines in the Ouchterlony tests demonstrate a number of antigens to be common to both hydranth and stem. The immunoelectrophoretic patterns showed that at least six of the hydranth antigens had electrophoretic mobilities comparable to those of six stem antigens. In addition to the similar antigens the tests with unabsorbed sera showed one antigen to be limited to the hydranth and at least two antigens limited to the stem.

Tests with absorbed anti-whole animal serum. In order to resolve further the antigenic differences between hydranth and stem extracts, absorbed serum (Rabbit 6) was used in Ouchterlony tests. Antiserum absorbed with hydranth extract still produced two distinct and one faint, diffuse precipitin lines with stem extracts. Antiserum absorbed with stem extract produced one precipitin line with hydranth extract. The absorbed sera failed to produce bands with the absorbing antigen. These results again indicate there are at least two antigenic substances restricted to stems and one restricted to hydranths.

Discussion

The results demonstrate that precipitating antibodies can be obtained against saline extracts of *Tubularia*. The agar diffusion tests show that a spectrum of precipitating antigens is present in stems and hydranths.

It is possible that some of these antigenic substances are not actually part of the tubularian tissues. In spite of the precautions described (see methods section), the antigens restricted to the stem extracts may be from organisms associated with the perisarc which is limited to the stem region. The antigenic differences may also be due to breakdown products of ingested food. The discussion presented here is subject to these reservations.

A number of antigens appear to be common to both hydranths and stems. It was previously reported (Morrill, 1958) that there were seven common antigens. Re-examination of Ouchterlony patterns has revealed that at least three antigens are common to both regions of the animal. In addition, one stem antigen has antigenic sites similar to those on two hydranth antigens. Immunoelectrophoretic experiments with anti-hydranth serum showed six antigens of hydranths and stems to have similar electrophoretic mobilities. This method resulted in distinct non-overlapping lines and should prove useful in future studies on the antigenic composition of this organism. Ouchterlony tests with non-absorbed and absorbed anti-whole animal sera indicate that at least two antigenic substances are limited to stems and one to hydranths.

With the establishment of antigenic relations between stems and mature hydranths further investigations need to be conducted to determine the antigenic relations between stems, regenerated hydranths, and hydranths at different stages of regeneration. The antigens need also to be characterized. Preliminary experiments show that antisera inhibit hydranth regeneration. Hydranth-specific and stem-specific antibodies should now be tested for inhibiting action on the regeneration of hydranths.

I wish to express my appreciation to Dr. Charles B. Metz for his guidance, encouragement, and interest during the course of this investigation.

Summary

1. The antigenic composition of hydranths and stems of *Tubularia crocea* has been studied by means of the precipitin ring tests, Ouchterlony agar gel diffusion tests, and immunoelectrophoresis.

2. Precipitin ring tests showed that antiserum against whole animals contained precipitating antibodies but failed to reveal antigenic differences between hydranths and stems.

3. Ouchterlony tests of anti-whole animal serum and saline extracts of hydranth and stem tissues revealed the following:

a. Four antigens common to both regions of the animal.
b. One stem antigen with hapten sites similar to those on two hydranth antigens.
c. Two antigenic substances limited to stems.
d. One antigenic substance limited to hydranths.
e. One stem antigen with at least two haptens—one in common with a hydranth antigen and one which was not related to any precipitating hydranth antigens.

4. Six stem antigens and six hydranth antigens had comparable electrophoretic mobilities.

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